CATECHOLAMINES IN THE HEMOLYMPH AND CUTICLE OF THE AMERICAN COCKROACH, *PERIPLANETA AMERICANA* (L.) AND THE MADEIRA COCKROACH, *LEUCOPHAEA MADERAE* (F.)

by

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I. INTRODUCTION

Exoskeleton

The exoskeleton is one of the most important structures contributing to the success insects have had in exploiting diverse terrestrial and aquatic environments. The cuticular exoskeleton, composed primarily of chitin, proteins, lipids, and stabilized by aromatic amino acid metabolites, provides support for the muscles and internal organs and protection from predators, pathogens and parasites. It also functions as a barrier against water loss thus preventing dehydration. The structure and composition of the exoskeleton confers the varied properties of light-weight, flexibility and strength, which has allowed insects to develop flight as an important means of locomotion for dispersion and colonizing new environments (Neville, 1975; Chapman, 1982).

One major disadvantage to the exoskeleton, however, is its inability to expand beyond certain physical limits. This periodically requires the digestion of parts of the old cuticle, secretion of a new, expandable cuticle and the shedding of the indigestible remains of the old one. The formation of new cuticle and the shedding of the old cuticle are a very crucial and highly complex sequence of biological events in insects. The molting cycle is initiated through the release of the prothoracotropin (PTTH), in response to neural commands for the

brain. PTTH is released in the hemolymph from the corpora allata and stimulates the prothoracic gland to produce the molting hormones, or ecdysones. 20-Hydroxyecdysone initiates apolysis and new cuticle formation and regulates most processes associated with ecdysis. The insect separates the epidermis from the cuticle (apolysis), and secretes a gel or molting fluid into this space. The molting fluid, which contains proteolytic, chitinolytic and lipolytic enzymes, digests the untanned layers of old cuticle. Most of the old cuticle is digested except for the thin outer layer that consists of epicuticle, exocuticle and remnants of undigested endocuticle. The digested materials are then reabsorbed into the insect to conserve important metabolites. Secretion of new cuticle begins after apolysis with the new epicuticle preventing digestion by the molting fluid. This allows the laying down of new cuticle and the digestion of old cuticle to occur simultaneously.

Ecdysis, the shedding of the exuvium or remnants of old cuticle, now occurs by the insect rupturing the old cuticle at the ecdysial suture and pulling itself out. After ecdysis the newly secreted cuticle is expanded to allow for growth. It then begins to stabilize and harden. This process called tanning or sclerotization is thought to involve the dehydration and cross-linking of the cuticle's chitin-protein matrix with catechols and quinones. Sclerotization is activated by the hormone bursicon which is released at ecdysis or shortly thereafter. Bursicon is thought to regulate the metabolism of catecholamines from tyrosine, and the expansibility of newly secreted cuticle after ecdysis.

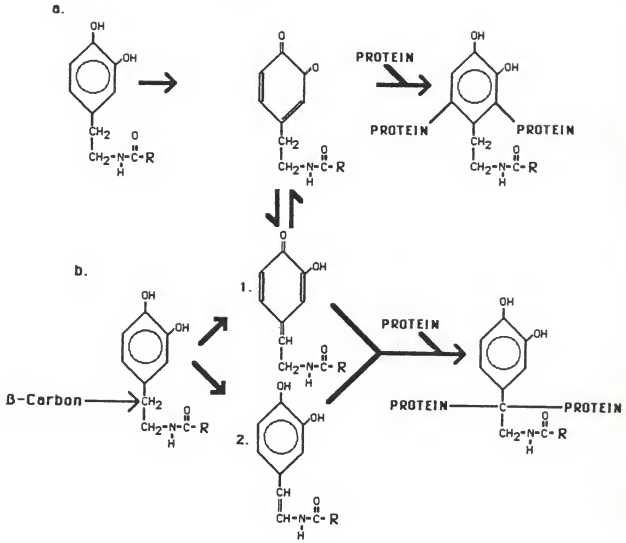
A few species may also deposit minerals, such as calcium or magnesium, in the exoskeleton to help with the stabilization of the cuticle. Cuticular sclerotization is a very precisely defined sequence of metabolic events for individual species at different development stages before and after ecdysis. A failure in any one of these events could damage or destroy the insects ability to develop to a more mature stage.

Catecholamines are converted into cross-linking metabolites for sclerotization. There are two current hypotheses by which the cross-linking of cuticular proteins with catecholamines occur. One is called quinone tanning, where the proteins become directly linked to the aromatic ring of a catecholamine quinone, such as N-acetyldopamine quinone (Fig. 1a). The other hypothesis called B-sclerotization, states that bonding occurs between the proteins and the catecholamine, such as N-B-alanyldopamine, at the B-carbon of the side chain (Andersen and Barrett, 1971; Andersen, 1979; Sugumaran and Lipke, 1983)(Fig. 1b). Both quinone and B-sclerotization are thought to occur in the same insect cuticle, but to varying degrees. Apparently not all catecholamines in the cuticle are covalently bound to the chitin-protein matrix. A relatively large amount of catecholamines are extractable from the cuticle, using relatively mild conditions, after the stabilization of the cuticle is nearly complete (Hopkins et al., 1984). These catechols may act as filling material to further help stabilize the cuticle.

A third type of cuticular protein cross-linking is the bityrosine mechanism. It involves the cross-linking of hinge protein through bi or

Figure 1. The two theories of sclerotization

a. Catecholamine metabolism during quinone tanning.
b. Catecholamine metabolism during B-sclerotization.
    1. Quinone methide
    2. 1-2 dehyrdo catechol



R= $-CH_3$ N-acetyldopamine

R= $-CH_2-CH_2-NH_3$ N-ß-alanyldopamine

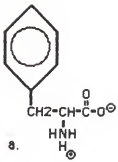
ter-tyrosyl bridges to form the highly elastic cuticle resilin. Tyrosinase or peroxidase activates this process (Andersen, 1966). Bityrosine has also been recovered from proteins of prepuparial cuticle of Sarcophaga bullata Sugumaran et al., 1982. No catecholamines or metabolites are thought to be involved in this type of cross-linking.

Quinones of NADA and NBAD have been shown to cross-link callipho-rin, drosophilin, manducin and extractable cuticular proteins from the last instar and early pupae of M. sexta (Grun and Peter, 1983). NADA and NBAD did not bind these proteins unless oxidized by tyrosinase to their corresponding quinones.

Metabolism of tyrosine to catecholamines

Catecholamines are the major metabolites for the sclerotization, melanization and stabilization of insect cuticle (reviewed by Andersen, 1979; Brunet, 1980; Hillerton and Vincent, 1979). However, some insects including Musca autumnalis (face fly), may predominantly use calcium and other minerals to stabilize their puparial cuticle rather than catechols (Fraenkel and Hsaio, 1967; Darlington et al., 1983; Roseland et al., 1985). Catecholamines and their corresponding quinones are derived from the amino acid precursor tyrosine (Fig. 2b). Tyrosine can be assimilated from the insect's diet or arise through the hydroxylation of phenylalanine (Fig. 2a). Tyrosine and phenylalanine can often be stored in hemolymph proteins, peptides or other conjugates until they are needed for catecholamine metabolism. In newly ecdysed and mature adults

Figure 2. Chemical structures of catechols, their precursors and metabolites. a = phenylalanine, b = tyrosine, c = DOPA, d = dopamine, e = N-acetyldopamine, f = N-B-alanyldopamine, g = dopamine-3-O-sulfate, h = N-acetylnorepinephrine, i = N-B-alanylnorepinephrine, j = protocatechuic acid.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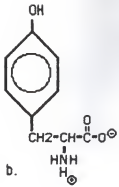
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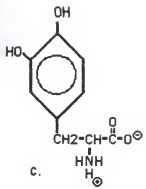
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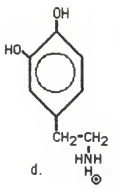
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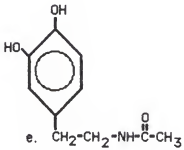
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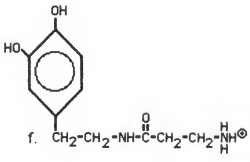
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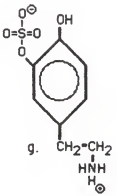
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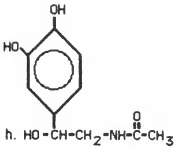
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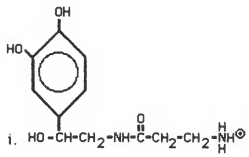
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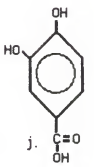
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of Periplaneta americana, tyrosine has been found to be a major metabolite of phenylalanine (Murdock et al., 1970a). Phenylalanine is rapidly converted into tyrosine in newly ecdysed and mature adults.

The catecholamine dopamine (Fig. 2d) is most commonly linked to acetate or B-alanine on the amino group to form N-acetyldopamine (NADA) (Karlson and Sekeris, 1962) (Fig. 2e) or N-B-alanyldopamine (NBAD) (Hopkins et al., 1982; 1984) (Fig. 2f) respectively in insects during formation of new cuticle. These catecholamines and others may be conjugated on a ring oxygen with glucose, phosphate or sulfate for possible protection or transport of these molecules (reviewed Brunet, 1980). Cuticle hardness and pigmentation appears to depend in part on the types and amounts of catecholamine synthesized and how they are incorporated into various regions of the exoskeleton (Hopkins et al., 1982; 1984). NADA has been associated with colorless hard cuticle, NBAD with hard brown cuticle and dopamine with melaninized cuticle (Hopkins et al., 1982; 1984; Kramer et al., 1984).

Catecholamines in Periplaneta americana

Dopamine-3-O-sulfate was isolated in whole body extracts of newly ecdysed P. americana (Bodnyark and Brunet, 1974)(Fig. 2g). Newly ecdysed nymphs and adults had the highest levels of dopamine-3-O-sulfate. By 5 hours after ecdysis the amount of dopamine-3-O-sulfate had declined by 70%. Within 16 hours after ecdysis only trace amounts of dopamine-3-O-sulfate were detected. Little or none of the sulfate ester

was present in the cuticle. In vertebrates, conjugated sulfates have usually been associated with excretory products (Imai et al., 1970; Merits et al., 1973; Merits, 1976). This appears not to be the case for dopamine-3-O-sulfate as it is involved in the sclerotization of P. americana cuticle. The sulfate conjugate may protect dopamine from oxidation (Bodnyark and Brunet, 1974).

N-Acetyldopamine has been reported in P. americana (Mills et al., 1967; Murdock et al., 1970; Bodnyark et al., 1974). Mills reported that crude homogenates of cockroach enzymes were capable of synthesizing N-acetyldopamine from tyrosine, while Murdock et al. (1970) observed that radiolabeled tyrosine injected into newly ecdysed adults was metabolized to N-acetyldopamine. Bodnyark et al. (1974) isolated two esters of NADA from homogenates of newly ecdysed nymphs, one being NADA-3-O-phosphate and the other NADA-3-O-sulfate. The radioactive NADA moiety of these esters was found in the cuticle.

Several other catechols have been identified in exuvia of P. americana including protocatechuic acid (3,4-dihydroxybenzoic acid), protocatechualdehyde (3,4-dihydroxybenzaldehyde), 3,4-dihydroxyphenylethanol and 2-hydroxy-3,4-dihydroxyacetophenone (Atkinson, 1973). The ootheca of P. americana has been found to contain only protocatechuic acid (3,4 dihydroxybenzoic acid) (Fig. 2j), as reported by Pryor (1940). It is stored in the left colleterial gland as a 4-O-B-glucoside (Brunet and Kent, 1955) and is thought to be the cross-linking agent of the protein in the ootheca.

The tanning or sclerotization of insect cuticle is an vital physiological process in the development of an insect. A better understanding of the metabolic pathways involved in this process might lead to new information in developmental biology and provide insight for the discovery of new types of insect growth regulators that interfere with cuticle formation during the molting cycle.

Objectives

The two main objectives in this study were

1) to determine the catecholamines and their metabolites in the cuticle and hemolymph of the cockroaches Periplaneta americana and Leucophaea maderae during tanning or sclerotization. Developmental changes in cuticle and hemolymph titers during ecdysis and cuticular stabilization in nymphal stages and adults were also studied.

2) To survey the catecholamines in the hemolymph of several other species of cockroaches during the tanning or sclerotization of new cuticle.

II. MATERIALS AND METHODS

Insect colonies

Stock colonies of the cockroaches, L. maderae (Madeira), P. americana (American), Blatta orientalis (Oriental), Blaberus craniifer, Blattella germanica (German) including the mutant pale body, and Gromphadorhina portentosa (Madagascar) were reared and maintained at a temperature of $28^{o}\pm2^{o}C$, and a photoperiod of 16 hours light and 8 hours dark. The cockroaches were kept in containers with wood shavings and water and Purina laboratory "Lab Chow" were provided ad lib. The B. germanica mutants were a gift from Dr. Mary Ross, Department of Entomology, Virgina Polytechnical Institute, Blacksburg, Virginia.

Last stage nymphal cockroaches were selected from the colonies, sexed, and placed in individual rearing cages made from square plastic petri dishes with food and water given ad lib. Individuals were checked daily for variations in eye color. P. americana, L. maderae and B. orientalis eye color changes from a shiny black to a dull grayish blue at apolysis. The variation in eye color is due to the change in the refraction of light as the the old cuticle separates from the insect during apolysis. The eye color becomes a brighter blue as the cockroach nears ecdysis (Flint and Patton, 1959; Wirtz and Hopkins, 1977).

Cockroaches were collected for analysis at different ages during the last nymphal period through several weeks after adult ecdysis. However, most of the cockroaches were selected during the time interval from apolysis to 48 hours after ecdysis.

Hemolymph sampling

Several methods for collecting measured volumes of unclotted hemolymph were investigated.

Method 1.

Insects anesthetized with carbon dioxide had an antenna clipped to cause bleeding. Hemolymph was collected on a glass slide, then placed on dry ice. The quick freezing of the hemolymph prevented clotting while a crystal of phenylthiourea prevented blackening due to phenoloxidase activity.

The collected hemolymph was then diluted 1 part hemolymph to 9 parts of a 1.2 M HCl solution containing an internal standard of 120 nanograms (ng) of dihydroxybenzylamine (DHBA) and 5 mM ascorbic acid, the latter added to protect the catechols from oxidation.

This method proved to be unsatisfactory due to condensation of water vapor on the slide and the resulting error in volume measurements.

Method 2.

Cockroaches were anesthesized and quick-frozen in a dry ice powder and stored at $-20^{o}C$ until the samples could be collected and analyzed.

The insects were placed in a desiccator (5 min.) to thaw and prevent water condensation (Hopkins and Wirtz, 1976). The appendages (wings, legs and antenne) of three to four neck-ligated cockroaches were removed, and incisions were made in the integument in the cervical membranes. The insects were then placed head down in brass screen cylindrical tubes fitted inside conical 50 ml centrifuge tubes. Centrifugation at low speeds was sufficient to collect the hemolymph in the bottom of the tube. The hemolymph was then diluted with the internal standard (1-10 dilution as in Method 1). A potential problem with this method was the possible contamination of the hemolymph with various other tissues, e.g. fat body, during centrifugation.

Method 3.

Cockroaches were anesthesized and quick-frozen in dry ice powder and stored at -20°C until analysis. After thawing in a desiccator (5 min), the insects were placed in a spring steel clip with light pressure being applied to the abdomen. The insect was suspended head down for 1 minute after which the front coxa of the left leg was severed and the hemolymph collected with micropipettes (5 or 10 microliters). Internal standard was added to the hemolymph as in Method 1. Samples were centrifuged at 6500g for 10 minutes and the supernatant was removed for analysis.

The freezing and thawing of the insects may have caused cells and tissues to rupture and thus release their contents into the hemolymph.

Method 4.

Method 4 was similar to Method 3 except that live insects were used. The insects were anesthesized and placed in the spring clip as previously described. A micropipet with a crystal of phenylthiourea in it was used to collect the hemolymph from the coxa. The major problem of this method was the clotting of hemolymph.

Method 3 was the method of choice in this experiment. It gave us a readily available source of unclotted hemolymph and we were able to store insects until analysis could be conducted. Preliminary investigation indicate that the freezing and thawing of the insects does not cause any appreciable errors in method 3 in hemolymph catechol concentrations (See Appendix A).

Cuticle sampling

Integument was dissected from the cockroach and adhering muscle and fat body were dissected from the integument which was then cut into two equal pieces. One piece was rinsed with distilled water, while the other was scraped to remove the epidermis and then rinsed with distilled water. The pronotum was the major area of cuticle analyzed, but the head capsule, thoracic and abdominal cuticles were also analyzed in some species. The pieces were blotted on absorbent tissue, weighed (0.5 to 5.0 milligrams) and then homogenized in a ground glass tissue grinder in 300 ul of 1.2 N HCl and 5 mM ascorbic acid containing either DHBA (12

ng) or with alpha methyl dopa (120 ng). The homogenate were centrifuged at 6500 g's for 10 minutes and the supernatant removed for liquid chromatography with electrochemical detection (LCEC) analysis.

Whole body sampling

Whole animals of different stages were homogenized in 80% aqueous methanol containing 5mM ascorbic acid as described by Wirtz and Hopkins (1974; 1977). Each insect was homogenized in 15 ml ice cold extraction medium for 2 minutes at high speed in a stainless steel vessel (Omni-mixer, Sorval). The homogenate was concentrated for thin-layer chromatography (TLC).

Hydrolysis and recovery of catechols

Aliquots of hemolymph or cuticle supernatants (250 ul) were heated at 100°C under nitrogen for 10 minutes to hydrolyze conjugates. Samples of the hydrolyzed and unhydrolyzed extracts (50 to 100 ul) were prepared for analysis by liquid chromatography with electrochemical detection (LCEC) according to Hopkins et al. (1982; 1984). Catechols were adsorbed on alumina by the methods of Murdock and Omar (1981).

Samples were placed in 1.5 ml plastic centrifuge tubes containing 1 ml of a tris-buffer (pH 8.0) and 23-25 mg of an alumina-EDTA mixture (2:1) and then shaken for 15 minutes. The supernatant was discarded and the alumina pellet with the adsorbed catechols were washed by resus-

pending in tris-buffer (1 ml). The tubes were shaken for 5 minutes and then centrifuged for 5 minutes at 6500 g's. The supernatant was discarded and the pellet was resuspended in 0.1 ml of 1 M acetic acid (pH 1.9) to extract the catechols from the alumina as the lowered pH released the catechols into the 1M acetic acid. The tubes were shaken for 15 minutes and then centrifuged for 15 minutes at 6500 g's. The supernatant were then stored at 4-5 ^{o}C, until LCEC analysis.

LCEC analysis of catechols

Samples were analyzed on a Bioanalytical Systems LC 304 chromatograph with an LC 4A electrochemical detector operated at +0.72 volts, using a reverse phase ODS 5 micrometers x 25 centimeters spherical particle column (Hopkins et al. 1982). The primary mobile phase for separation of catechols from hemolymph samples consisted of distilled water, methanol (17.5%), sodium octyl sulfate (SOS) (3.4×10^{-1} mM), and sodium EDTA (9.6×10^{-2} mM) buffered with H_3PO_4 to pH 3.1. The primary mobile phase for separation of catechols from cuticle samples consisted of distilled water, methanol (15%), SOS (1.6×10^{-1} mM), and sodium EDTA (9.0×10^{-2} mM) buffered with H_3PO_4 to pH 2.94. The retention times for the catechols separated in these primary mobile phases are shown in figure 3. Other mobile phases with different concentrations of the above materials were used to adjust the retention times of the catecholamines that had similar elution volumes due to column aging. A

Figure 3. LCEC elution profile of catecholamine standards used for both hemolymph and cuticle samples. (a.) hemolymph standard, octyl-sulfate mobile phase. (b.) cuticle std., octyl-sulfate mobile phase.

a.
NE
DOPA
DHBA
DA
DOPAC
DHBZ
NBAD
NADA
0
5
10
15
TIME IN MINUTES
b.
NE
DOPA
NBAINE
NANE
DA
AMDOPA
NBAD
NADA
0
5
10
15
TIME IN MINUTES
DETECTOR RESPONSE (nA)

mobile phase using sodium lauryl sulfate (SLS) as the ion pairing agent was used to change the retention times to further confirm the identity of the catecholamines. The latter mobile phase consisted of distilled water, acetonitrile (49%), SLS (1.6^{-1} mM), and EDTA (5.9×10^{-2} mM) buffered with H_3PO_4 at pH 3.03.

The retention times of the catecholamine standards in both octyl and lauryl sulfate were used for comparison to unknown catechols in hemolymph and cuticle extracts (Hopkins et al., 1982). Quantities of individual catecholamines were calculated by comparing peak heights with that of the internal standard in each extract, then correcting for recoveries established using standard compounds. The percent conjugation of each of the catecholamines was calculated by subtracting the amount of the catecholamine in the unhydrolyzed sample from the amount of the catecholamine found in the hydrolyzed sample. This value was divided by the hydrolyzed sample (total catechols) and multiplying by 100.

Source of standard compounds

Dopamine, tyrosine, 3,4-dihydroxyphenylalanine (DOPA), N-acetyldopamine (NADA), norepinephrine (NE), 3,4-dihydroxyphenylacetic acid (DOPAC), DHBA, and alpha-methyl-DOPA were from commercial sources, e.g. Aldrich Chemical Company and Sigma Chemical Company. N-B-Alanyldopamine (NBAD) was prepared as by coupling dopamine with N-a-t-butyloxy-

carbobenzoyl-b-alanine-N-hydroxy-succinimide ester in potassium tetraborate buffer and deblocking the conjugate in 0.1N HCl according to Hopkins et al. (1982) and Aso et al. (1984). Identity was confirmed by LCEC and ultraviolet spectroscopy. Purity was > 99 percent. N-B-alanylnorepinephrine (NBANE) was synthesized by a similar procedure. Tyrosine-O-sulfate, dopamine-O-sulfate and DOPA-O-sulfate were synthesized by sulfation at low temperatures in concentrated sulfuric acid (Tallen et al., 1955; Jenner and Rose, 1973). N-Acetylnorepinephrine (NANE) was synthesized by acetylating NE with acetic anhydride in sodium tetraborate according to Andersen (1980).

Two-dimensional chromatographic mapping of radiolabeled tyrosine metabolites

Last nymphal instars of L. maderae and P. americana were anesthetized with carbon dioxide and then injected with 2.5 uCi of radioactive 14C-U-tyrosine or 5.0 uCi of sulfate-35S between the 3rd and 4th dorsal abdominal tergites. The insects were sacrificed at three different developmental stages: feeding last instars (6 hours after injection), pharate adults after apolysis (approx. 72 hours after injection), and adults after ecdysis (3-12 hours after ecdysis, greater than 72 hours after injection). Samples of hemolymph and whole body were taken at intervals during the last molting cycle for chromatographic analysis of tyrosine metabolites and their conjugates.

Hemolymph and whole body extracts were analysized before and after hydrolysis (1.2N HCl for 10 min. at 100 ^{o}C to release catechols from their conjugates). Two-dimensional separation was done by electrophoresis and thin-layer chromatography (TLC) on cellulose sheets (Eastman, 20 x 20 cm). Electrophoresis was conducted for 2.7 to 5 hours at pH 1.9 (8% acetic acid, 2% formic acid) and 200 V per plate. This procedure was followed by TLC with a butanol:pyridine:acetic acid:water mixture (15:10:3:12). In some instances, the plate was then developed a second time by TLC in the same direction as electrophoresis to enhance separation of certain catecholamine conjugates.

Two or more chromatographic plates of each sample (both hydrolyzed and unhydrolyzed) were prepared. Some plates were sprayed with different reagents (ninhydrin or diazotized sufanilic acid) to locate amino acids, phenols and catechols, respectively, and compared to standard compounds chromatographed the same way. Radioactive metabolites were located by autoradiography with x-ray film or by a Vanguard 880 Automatic Chromatogram Scanner. Autoradiography was performed with Kodak No Screen X-ray film being laid on the cellulose plates and covered. These films were kept in light tight boxes at freezer temperature for 60-100+ days and then developed to locate the radioactive areas. Some spots from sprayed plates were scraped and the radioactivity determined on a Beckman LS-100 Liquid Scintillation Counter.

To confirm the identity of the radiolabeled metabolites, the radioactive areas from unsprayed plates were located as previously described and the cellulose removed and extracted with 80% aqueous methanol containing 5 uM ascorbic acid solution. Aliquots were then analyzed by LCEC either directly or after hydrolysis for 10 minutes under nitrogen at 100 ^{o}C.

LCEC identification of radiolabeled metabolites

Extracts of hemolymph from 14C-U-tyrosine injected cockroaches were hydrolyzed to release free catechols from their conjugates as described previously. These samples were then analyzed by LCEC to determine the catecholamines present in the extracted samples. Individual metabolites from other injections were then collected as they emerged from the column. Radioactivity in the aliquots were determined by a liquid scintillation methods to determine which compounds were derived from 14C-U-tyrosine.

Data handling

Data handling was accomplished using the software program "Multiplan" by Microsoft with a Z-100 Zenith/Heath computer. Data from the HPLC injections were recorded on chart recorder paper, research notebook, and computer diskette. The data stored were peak heights in milli-

meters, sample size (0.1 ml hemolymph or cuticle weight in grams), and voltage from detector.

Calculations

Catecholamine concentrations from each sample were calculated in the following manner: 1. a series of trials were done to determine the percent recovery of each catecholamine from alumina by comparing peak heights of the individual catecholamines to the internal standard to compute a ratio for samples recovered from alumina and standard sample. This recovery correction value was checked periodically at 3 month intervals. 2. A standard mixture of catecholamines of known concentrations was analyzed by LCEC each time a new batch of mobile phase was used to insure adequate separations of the compounds. 3. Catecholamine concentration was calculated by the equations listed on the next page.

Equations

$$\text{Ratio for Recovery of Catechols from alumina} = \frac{rIS}{rCAstd}$$

$$\text{Ratio for Standard Mixture} = \frac{aCAstd \times pIS}{aIS \times pCAstd}$$

Calculation of Catecholamine in sample =

$$\text{Catechol conc. in hemolymph or tissue} = \frac{rIS \times pIS \times aCAstd \times pCAs \times aISs \times f}{rCAstd \times pCAstd \times aIS \times pISs \times Y \times mwtCA}$$

Legend:

a = amount (ng)	p = peak height (mm)
CA = catechol	r = recovery (%)
f = dilution factor	s = sample
IS = Internal standard (DHBA)	std = standard
mwt = molecular weight	Y = weight (g) or volume (ml)

Sample Calculation

Standard Recovery of Catechols from alumina =

$$\frac{100 \text{ (Recovery of internal standard)}}{89 \text{ (Recovery of NADA)}} = 1.124$$

Ratio for Standard Curve =

$$\frac{424 \text{ ng NADA} \times 100 \text{ mm ht DHBA (std)}}{303 \text{ ng DHBA} \quad 40 \text{ mm ht NADA (std)}} = 3.498$$

Calculation of Cat in sample =

$$\frac{1.124 \times 3.498 \times 20 \text{ pNADAs} \times 108 \times 5}{40 \text{ pDHBAs} \times 0.01 \times 195.21} = 543.81 \text{ umoles NADA/liter}$$

Catecholamine concentrations in hemolymph and cuticle for each age group were calculated by taking the ratio of peak heights and weights of the standard mixture and multiplying by the ratio of the recovery of the internal standard over the recovery of the catecholamine in question. Sample concentrations are calculated by the ratio of peak heights of the sample and peak height of DHBA to the known weight of DHBA times the dilution factor since only 20 ul of the 100 ul sample are injected. This value is then divided by the amount of hemolymph in ml of the sample. This is divided by the molecular weight of the cat in question to give the answer in umoles per liter. Mean $\pm$ S.E.M. of each group sampled were then stored.

III. RESULTS

Catechol metabolites in hemolymph during development

Periplaneta americana. Several catechols were identified by LCEC analysis in the hydrolyzed extracts of hemolymph of newly ecdysed nymphal and adult P. americana. They were dopamine (DA), N-acetyldopamine (NADA), N-B-alanyldopamine (NBAD), norepinephrine (NE), 3,4-dihydroxyphenylalanine (DOPA), 3,4-dihydroxyphenylacetic acid (DOPAC), 3,4-dihydroxybenzoic acid (DHBZ) and several unidentified compounds (Fig. 4a). An unidentified catechol (unknown I), dopamine, and NADA were the major catechols in P. americana hemolymph. Lesser amounts of NBAD, DOPA, DOPAC, NE AND DHBZ were also present.

Catechol titers in hemolymph and their percent conjugation were determined from one day after the last nymphal ecdysis of P. americana, to several months after adult ecdysis (Table 1). The catechols present in the nymphal and pharate adult stages were the same as those seen during adult ecdysis. Dopamine increased about 10-fold throughout the last instar, reaching a peak (approx. 1 mM) during the early pharate adult stage (Fig. 5). Approximately 80% of the dopamine was in the conjugate form, dopamine-O-sulfate (Table 1.). The dopamine concentration declined nearly 100-fold by 24 hours after ecdysis. The high-

Figure 4. LCEC profiles of catecholamines in the hemolymph of (a.) Periplaneta americana and (b.) Leucophaea maderae collected at ecdysis.

Dopamine	(DA)
Norepinephrine	(NE)
N-acetyldopamine	(NADA)
N-B-alanyldopamine	(NEAD)
3,4-Dihydroxyphenylalanine	(DOPA)
3,4-Dihydroxybenzoic acid	(DHBZ)
3,4-Dihydroxybenzylaldehyde	(DHBA)
3,4-Dihydroxyphenylacetic acid	(DOPAC)

a.
DETECTOR RESPONSE (nA)
NE
DOPA
DHBA
DA
DOPAC
DHBZ
UNK I
NBAD
NADA
A
0
5
10
15
TIME IN MINUTES

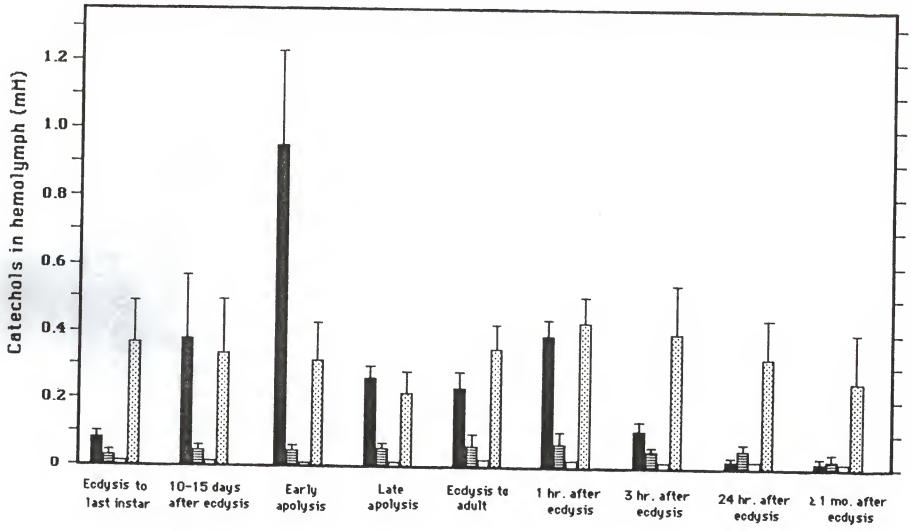
b.
DETECTOR RESPONSE (nA)
NE
DOPA
DHBA
DA
DOPAC
DHBZ
NBAD
NADA
A
0
5
10
15
TIME IN MINUTES

Table 1. Catecholamines (uM) in the hemolymph of last instar and adult of Periplaneta americana.

Stage	Oopamine	NAOA	NBAO	Unknown I	NE	OOPA
Last Instar						
Early (3-5 days)	80 ± 10	13 ± 5	3 ± 0.1	350 + 150	16 ± 5	7 ± 4
Middle (8-15 days)	380 ± 190	30 ± 15	3 ± 2	330 ± 120	14 ± 7	6 ± 3
Pharate Adult						
Early	950 ± 300 (83)	30 ± 15 (52)	7 ± 4 --	320 ± 100 (64)	31 ± 12	9 ± 4
Late	250 ± 70 (83)	20 ± 10 (55)	2 ± 1 --	200 ± 90 (65)	15 ± 11	4 ± 0.5
Adult						
Ecdysis	210 ± 80 (69)	40 ± 20 (80)	10 ± 7 (69)	320 ± 160 (89)	34 ± 10	19 ± 14
Postecdysis						
1 hr	410 ± 90 (82)	80 ± 60 (63)	14 ± 1 (66)	450 ± 110 (96)	33 ± 2	--
3 hr	130 ± 40 (86)	50 ± 10 (70)	7 ± 1 --	420 ± 210 (96)	12 ± 4	--
6 hr	40 ± 10 --	30 ± 20 --	8 ± 3 --	200 ± 130 --	18 ± 5	12 ± 5
12 hr	30 ± 5 (58)	50 ± 20 (53)	12 ± 10 (24)	200 ± 130 (95)	22 ± 16	8 ± 4
1 day	9 ± 5 (20)	40 ± 30 (35)	8 ± 5 --	325 ± 130 --	21 ± 16	5 ± 0.5
2 day	20 ± 10 (30)	30 ± 10 (43)	8 ± 2 (46)	250 ± 70 (88)	13 ± 10	9 ± 7
1 to 5 months	7 ± 6 (76)	15 ± 7 (76)	6 ± 7 (28)	270 ± 150 (93)	8 ± 3	9 ± 6

1. Number in parentheses gives percent conjugation. Mean ± S.E.M. from 3 to 8 samples.

Figure 5. Total major catechols (free and conjugated) in the hemolymph of Periplaneta americana during development. Units = mM (minimum of 5 samples + S.E.M.) ■ = dopamine, ▤ = NADA, □ = NBAD, and ⁘ = Unknown I.


Catechols in hemolymph (mM)
1.2
1.0
0.8
0.6
0.4
0.2
0
Ecdysis to last instar
10-15 days after ecdysis
Early apolysis
Late apolysis
Ecdysis to adult
1 hr. after ecdysis
3 hr. after ecdysis
24 hr. after ecdysis
≥ 1 mo. after ecdysis

est concentration of dopamine detected in the hemolymph, occurred at nymphal-adult apolysis .

Dopamine was the major identified catecholamine (approx. 0.21 mM) at nymphal-adult ecdysis, with a concentration about 5 times that of NADA (0.04 mM) and 20 times the concentration of NBAD (0.01 mM) (Fig. 5). Early after ecdysis, 70 - 80% of the dopamine was sequestered as a conjugate. Dopamine increased to 0.41 mM 1 hour later and then subsequently declined by 10-fold by 6 hours after ecdysis. Dopamine remained at a low level of approximately 0.01 mM (20-30% conjugation) from 24 hours through five months after ecdysis. Conjugation increased to earlier levels by 1 month after ecdysis. This differs dramatically from the last nymphal instar in which dopamine was being sequestered as a conjugate throughout the last nymphal instar.

The major electroactive unknown compound (Unk. I) in the hemolymph is very likely a catechol since it is a metabolite of tyrosine and is adsorbed on alumina at pH 8.6. Its titers remained relatively constant in hemolymph (approx. 0.3 mM) throughout the course of metamorphosis (Fig. 5) To determine if Unk. I was a tyrosine metabolite, cockroaches were injected with 14C-U-tyrosine and the hemolymph analyzed as previously described. Effluent was collected from the LC column at the retention times of Unk. I and aliquots were assayed for radioactivity by liquid scintillation counting. Unk. I was found to be radiolabeled, thus confirming it was a metabolite from 14C-U-tyrosine. Dopamine and

NADA were also radiolabeled demonstrating that they are metabolic products from tyrosine. The concentration estimates of the Unk. I indicated that this catecholamine is present in the hemolymph at high concentrations throughout ecdysis (Table 1, Fig. 5). Concentration estimates were made by averaging the percent recovery values for NADA (89%) and NBAD (100%) and using this number as the percent recovery value of Unk. I. This allowed comparison of Unk. I concentrations with those of NADA and NBAD. Unk. I remained at relatively high titers throughout the developmental time studied. In pharate adults the conjugation of Unk. I was determined to be 65%, while over 90% was conjugated in adults.

NADA, NBAD, NE, and DOPA did not show comparable fluctuations in their concentrations as did dopamine (Fig. 5). NADA was the third most abundant catechol and was present 1/30 to 1/3 the level of dopamine during apolysis-ecdysis. NADA and NE had higher hemolymph concentrations then NBAD or DOPA. 3,4-Dihydroxyphenylacetic acid (DOPAC) and 3,4-dihydroxybenzaldehyde (DHBZ) were only detected in trace quantities ($\leq$ 0.002 mM) and were not further studied.

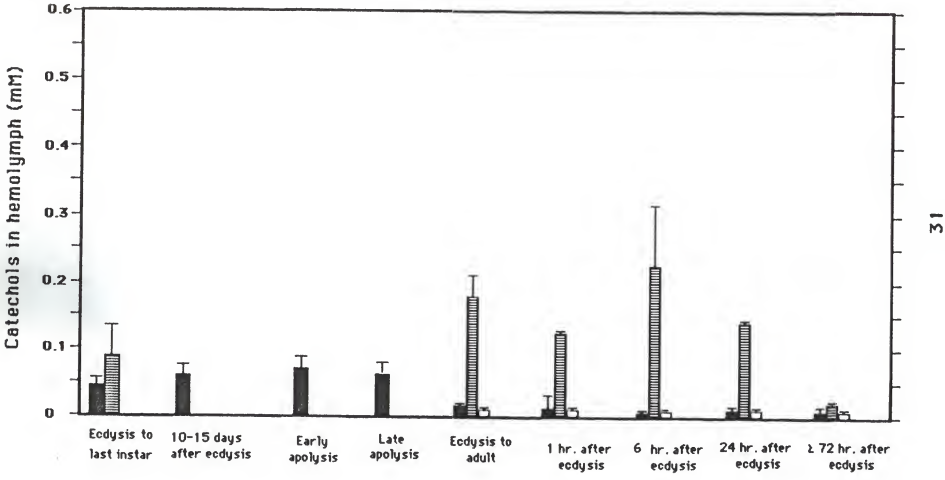
Leucophaea maderae. The catechols found in the hemolymph of L. maderae were similar to those in P. americana with the exception of the absence of Unk. I (Fig. 4b). The major catechol in the hemolymph was NADA (Table 2, Fig. 6). NADA reached peak titers (0.3 mM) at 6 hours after ecdysis, and then declined 10-fold by 72 hours after ecdysis.

Table 2. Catecholamine (uM) in the hemolymph of last instar and adult Leuchophaea maderae.

Stage	Dopamine	NADA	NBAD	NE	DOPA
Last Instar					
Ecdysis	40 ± 10	100 ± 15			
10 days	53 ± 32				
16 days	60 ± 30				
30 days	56 ± 11				
Ecdysis					
adult	15 ± 2	150 ± 40	5 ± 2	4 ± 2	8 ± 6
Postecdysis					
1 hr	35 ± 30	80 ± 6	15 ± 8	19 ± 13	7 ± 3
6 hr	13 ± 1	250 ± 100	8 ± 3	4 ± 2	13 ± 2
24 hr	19 ± 16	90 ± 9	14 ± 7	14 ± 10	7 ± 2
>72 hr	2.0 ± 0.4	20 ± 10	4 ± 1	18 ± 13	2 ± 1

Mean values ± SEM from 3 to 6 samples.

Figure 6. Total major catechols (free and conjugated) in the hemolymph of Leucophaea maderae during development. (minimum of 5 samples + S. E. M.) ■ = dopamine, ▤ = NADA, and □ = NBAD.


Catechols in hemolymph (mM)
0.6
0.5
0.4
0.3
0.2
0.1
0
Ecdysis to last instar
10-15 days after ecdysis
Early apolysis
Late apolysis
Ecdysis to adult
1 hr. after ecdysis
6 hr. after ecdysis
24 hr. after ecdysis
≥ 72 hr. after ecdysis

Dopamine, NBAD, NE, and DOPA had relatively low titers (0.01-0.02 mM) throughout the ecdysial period studied.

In nymphal L. maderae, dopamine was found to be stored as a conjugate of sulfate. The dopamine sulfate titers in L. maderae were much lower than those of P. americana . L. maderae stored 0.04-0.06 mM of dopamine sulfate throughout the last nymphal period (Table 2, Fig 6.).

Other species. Dopamine was found to be the major catecholamine in hemolymph in the majority of cockroaches species examined at nymphal-adult ecdysis. Blatta orientalis (0.25 mM), B. craniifer (0.16 mM), P. americana (0.21 mM), Blatella germanica (0.10mM) and Gromphadorhina portentosa (0.05 mM) all had high concentrations of dopamine in their hemolymph at ecdysis (Table 3). The B. germanica pale mutant had equal amounts of NADA and dopamine (0.01 mM). Only L. maderae was seen to have high concentrations of NADA (0.15m). Lower levels of NBAD were also present in the hemolymph of most cockroaches.

Table 3. Catecholamines (uM) in the hemolymph of several species of cockroaches at adult ecdysis

Species	Dopamine	NADA	NBAD
Periplaneta americana	210 ± 80	40 ± 20	10 ± 7
Blaberus craniifer	160 ± 20	30 ± 20	7 ± 3
Gomphodorhina portentosa	50 ± 15	14 ± 10	< 1
Blattella germanica			
Normal	100 ± 50	7 ± 2	< 1
Pale mutant	8 ± 2	10 ± 2	1 ± 2
Leuchophaea maderae	15 ± 2	150 ± 40	5 ± 2
Blatta orientalis	250 ± 100	45 ± 10	25 ± 10

Mean values ± SEM from three samples.

Catechol metabolites in cuticle

Periplaneta americana. Several free catechols were extracted from the cuticle of P. americana sampled after ecdysis (Fig. 7b). These catechols were dopamine, NADA, NBAD, DOPAC, and two new major electroactive metabolites, Unk. II and Unk. III, along with several other unidentified trace metabolites (Fig 7b).

Unk. II and Unk. III have tentatively been identified as N-acetylnorepinephrine (NANE) and N-B-alanylnorepinephrine (NBANE) (Fig. 2h, 2i) respectively, by LCEC analysis. Standards of NANE and NBANE were injected and their retentions times determined (Fig. 7a). Cuticle extracts were then compared (Fig. 7b) including co-injections with the standard mixture. The two new unknown compounds (Unknowns II and III) co-eluted with NANE and NBANE, respectively. Different mobile phases were used to demonstrate identical elution times of II and III to those of NANE and NBANE, respectively. This further confirmed that Unk. II is NANE and Unk. III is NBANE.

In new cuticle of pharate adult P. americana , there were no detectable free catechols present. At ecdysis, only a small amount of NBANE (approx. 0.11 mM/gram) was detected (Table 4, Fig. 8). NBANE was the major catecholamine found in cuticle during the first 48 hours after ecdysis, increasing 24-fold from ecdysis and reaching a peak (2.45 mM/gram) at 24 hours after ecdysis. NBAD was generally the next most abundant, (approx. 2.1 mM/gram) experiencing a 10-fold increase between

Figure 7. LCEC analysis of free catecholamines extracted from cuticle samples at ecdysis. (a.) Standard. (b.) Periplaneta americana (c.) Leucophaea maderae.

Dopamine	(DA)
Norepinephrine	(NE)
N-acetyldopamine	(NADA)
N-B-alanyldopamine	(NBAD)
N-acetylnorepinephrine	(NANE)
N-B-alanylnorepinephrine	(NBANE)
Alpha-methyldopa	(AMDOPA)
3,4-Dihydroxyphenylalanine	(DOPA)

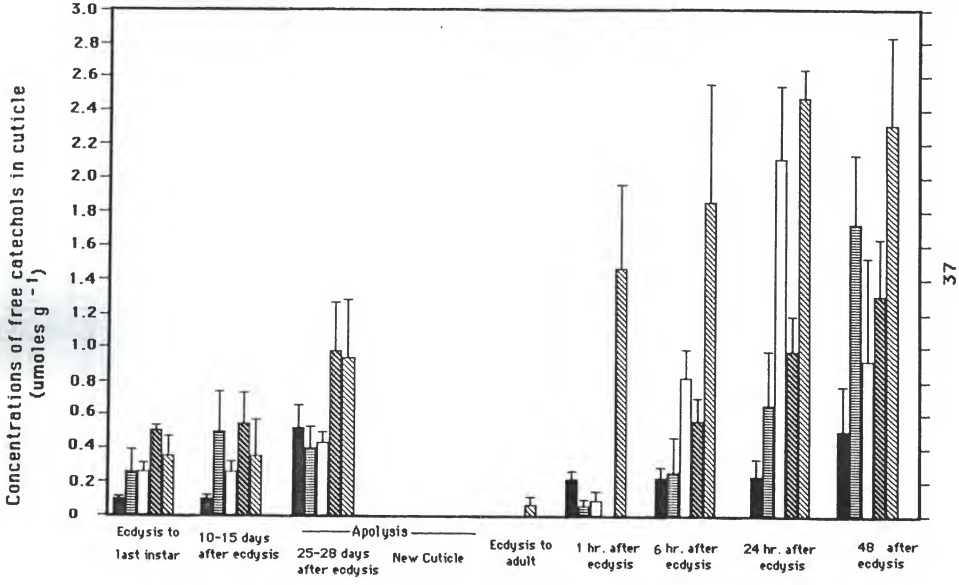
DETECTOR RESPONSE (nA)
a.
b.
c.
NE
DOPA
NBANE
NANE
DA
AMDOPA
NBAD
NADA
0
5
10
15
TIME IN MINUTES

Table 4. Catecholamine concentrations (nmoles/gram) in the cuticle of last instars and adults of Periplaneta americana.

Time after ecdysis (days)	Dopamine	NADA	NBAD	NANE	NBANE
Last instar					
Early (3 days)	107 ± 40	220 ± 100	220 ± 40	510 ± 60	380 ± 100
Middle (8-15 days)	--	480 ± 260	220 ± 40	580 ± 190	380 ± 240
Late (23-25 days)	570 ± 125	400 ± 100	470 ± 80	910 ± 150	870 ± 170
Pharate Adult	0	0	0	0	0
Ecdysis	0	0	0	0	105 ± 30
0.13	250 ± 50	100 ± 60	150 ± 60	0	1540 ± 440
0.45	190 ± 40	170 ± 150	410 ± 110	350 ± 80	1840 ± 750
1 day	400 ± 40	580 ± 230	2140 ± 550	750 ± 150	2450 ± 150
2 days	620 ± 350	2000 ± 400	1230 ± 500	1330 ± 340	2200 ± 440

Mean values ± S.E.M. from 3 to 5 samples.

Figure 8. Major catecholamines (free) in the cuticle of *Periplaneta americana* during development. (minimum of 5 samples + S.E.M.) ■ = dopamine, ▤ = NADA, □ = NBAD, ▨ = NANE, and ▨ = NBANE.


Concentrations of free catechols in cuticle (umoles g -1)
3.0
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
Apolysis
Ecdysis to last instar
10-15 days after ecdysis
25-28 days after ecdysis
New Cuticle
Ecdysis to adult
1 hr. after ecdysis
6 hr. after ecdysis
24 hr. after ecdysis
48 after ecdysis
37

1 and 48 hours after ecdysis, followed by NADA (2.0 mM/gram), except at 48 hours when NADA was similar in concentration to NBAD. NADA exhibited a 20-fold increase during the same period. NANE was not detectable in cuticle until 6 hours after ecdysis, but it rose to a level approximating that of NBAD at 48 hours after ecdysis (1.3 mM/gram). NANE increased about four-fold between 6 hours and 48 hours after ecdysis. Dopamine was also present in the cuticle, but at lower levels (0.6 mM/gram) and did not have such a dramatic increase.

Catechol concentrations were lower in larval cuticle, with NANE and NBANE being the most abundant. NANE, NBANE, NBAD, and NADA all increased about two-fold between 3 days after the last nymphal ecdysis to the onset of apolysis with the old pharate adult cuticle being analyzed for this value (Table 4, Fig. 8).

Leucophaea maderae. In L. maderae the free catecholamines present in the cuticle samples were the same as with P. americana (Fig. 7c). The concentrations were less, but the catechols increased throughout the time studied with NBANE being the most abundant (Table 5).

Table 5. Catecholamine concentrations (nmoles/gram cuticle) in the cuticle of adult Leucophaea maderae.

Time after ecdysis (hrs)	NADA	NBAD	NANE	NBANE
1	8 ± 3	9 ± 4	--	26 ± 10
3	14 ± 5	33 ± 12	--	61 ± 20
24	22 ± 13	40 ± 10	163 ± 30	452 ± 90
72	258 ± 70	60 ± 20	260 ± 80	2000 ± 1000

Mean values ± SEM from 3 to 5 samples.

Mapping of radiolabeled tyrosine and sulfate metabolites

Radioisotope studies of tyrosine metabolites and their conjugates were conducted on P. americana and L. maderae using 14C-U-tyrosine and 35S-sulfate. Hemolymph and whole body extracts (before and after mild acid hydrolysis) were prepared as described in the Methods section and were mapped by two-dimensional electrophoresis and thin-layer chromatography (TLC), with the detection of labeled compounds by autoradiography. A set of standard compounds was mapped for comparison with labeled metabolites. The location and numbering of the standard compounds on the chromatograms were as follows; I tyrosine, II dopamine-3 or 4-O-sulfate, III tyrosine-sulfate, and IV dopa-O-sulfate (Fig. 9).

The unhydrolyzed hemolymph extracts from last nymphal instars (before apolysis) of P. americana and L. maderae were found to inccrporate the radioactivity from tyrosine-U-C-14 into two areas on the cellulose plates as determined by autoradiography (Fig. 10a). Radiolabeled metabolite I corresponded to the free tyrosine standard. Radiolabeled metabolite II (unknown IV) was unidentified and was electrophoretically neutral at pH 1.9 and slightly more mobile in the TLC mobile phase than free tyrosine. Hydrolyzed samples of hemolymph (1.2N HCl at 100° for 10 min.) showed the disappearance of metabolite II and the tyrosine spot became more intense indicating that metabolite II was destroyed by hydrolysis. Extracts of whole body and hemolymph (hydrolyzed and unhy

Figure 9. Two-dimensional mapping of synthesized standards and free tyrosine.

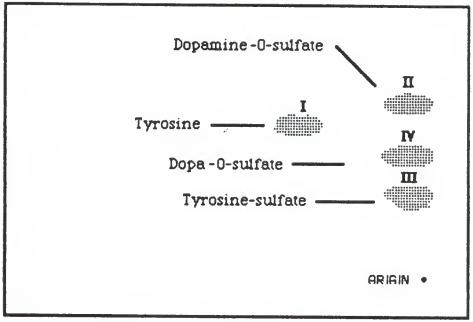
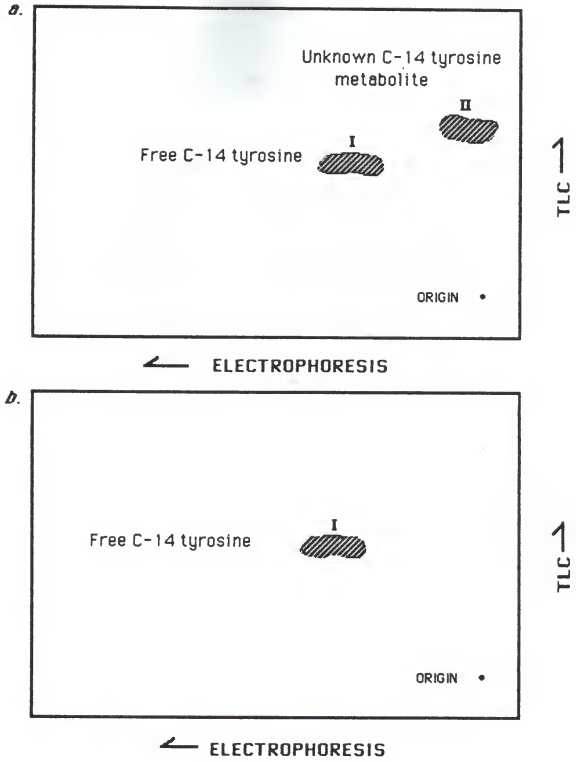

Dopamine-O-sulfate
II
I
Tyrosine
IV
Dopa-O-sulfate
III
Tyrosine-sulfate
ORIGIN
TLC
ELECTROPHORESIS

Figure 10. Analysis of C-14 tyrosine metabolites in last nymphal instar hemolymph and whole body extracts of Periplaneta americana, separated by 2-dimensional mapping. (a.) unhydrolyzed. (b.) hydrolyzed. Similar results are obtained with last instar Leucophaea maderae hemolymph.


a.
Unknown C-14 tyrosine metabolite
II
Free C-14 tyrosine
I
TLC
ORIGIN
ELECTROPHORESIS
b.
Free C-14 tyrosine
I
TLC
ORIGIN
ELECTROPHORESIS

drolyzed) from both P. americana and L. maderae exhibited the same fingerprint of two radioactive areas.

Fingerprint analysis of hemolymph and whole body extracts (unhydrolyzed) of S-35 sulfate injected P. americana (before apolysis) showed that the radioactivity was almost totally incorporated into a single radiolabeled zone (Fig. 11a, metabolite II) that had similar mobility to Unk. IV (metabolite II), from the ^{14}C-tyrosine injected insects. The S-35 labeled area disappeared in both the hemolymph and whole body extracts after acid hydrolysis (Fig 11b).

In P. americana pharate adults that were injected with C-14 tyrosine, both hemolymph and whole body extracts (unhydrolyzed) again had two major areas of radioactivity on the x-ray film (metabolite I, metabolite II, Fig. 12a, 13a). Whole body also exhibited four small rather faint areas of radioactivity (Fig. 13a metabolite III, IV, V, and VI). The major zones of radioactivity were identical to those observed in last nymphal instar hemolymph and whole body samples, Unk. IV (metabolite II) and tyrosine (metabolite I). Hydrolysis of both the hemolymph and whole body samples yielded only one major radioactive zone, that of free tyrosine (Fig. 12b, 13a). The results show that there is at least one tyrosine metabolite in the hemolymph and five tyrosine metabolites in the whole body samples. Hemolymph hydrolysis sample results demonstrate a possible tyrosine conjugate.

Figure 11. Analysis of S-35 metabolites in last nymphal instar hemolymph and whole body extracts of Periplaneta americana by 2-dimensional mapping. (a.) unhydrolyzed. (b.) hydrolyzed. ▨ = radiolabeled, and ▦ = non-radiolabeled.

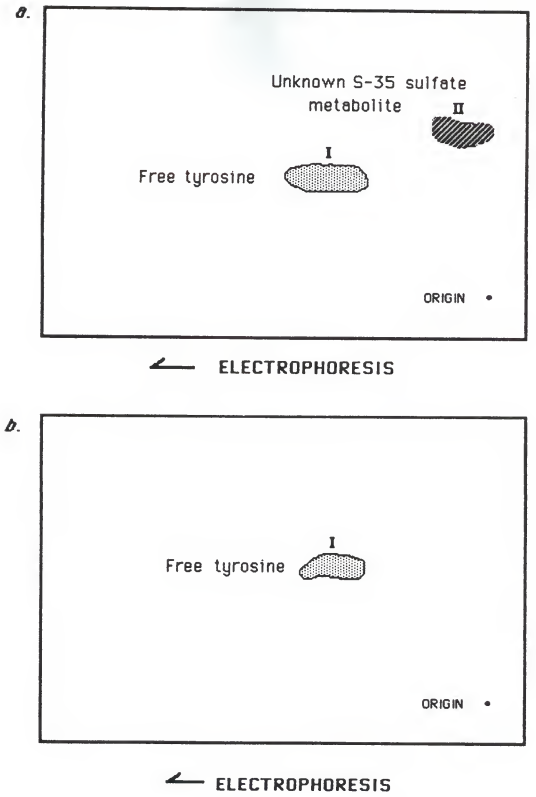

a.
Unknown S-35 sulfate metabolite
II
I
Free tyrosine
ORIGIN
TLC
ELECTROPHORESIS
b.
I
Free tyrosine
ORIGIN
TLC
ELECTROPHORESIS

Figure 12. Analysis of C-14 tyrosine metabolites in pharate adult hemolymph extracts of Periplaneta americana separated by 2-dimensional mapping. (a.) unhydrolyzed. (b.) hydrolyzed.

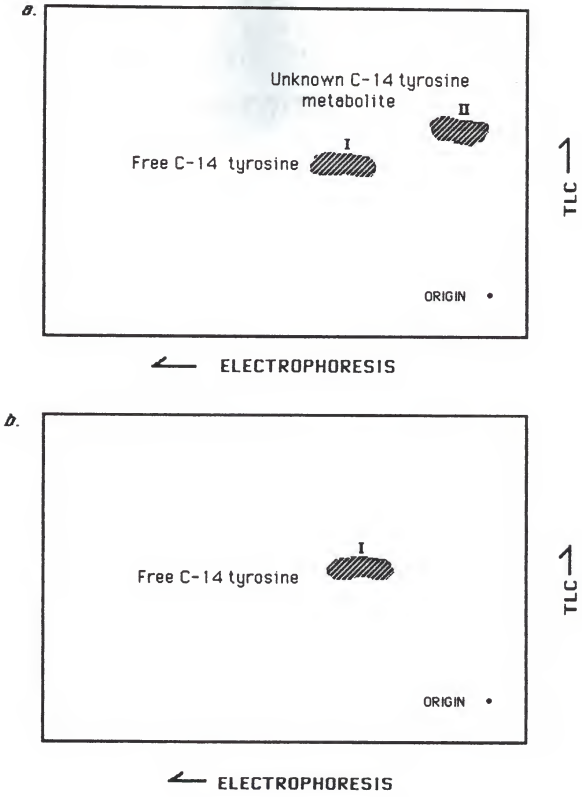
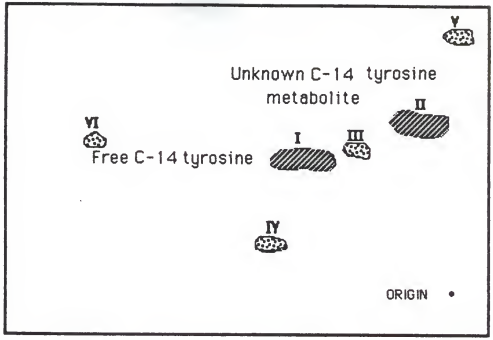
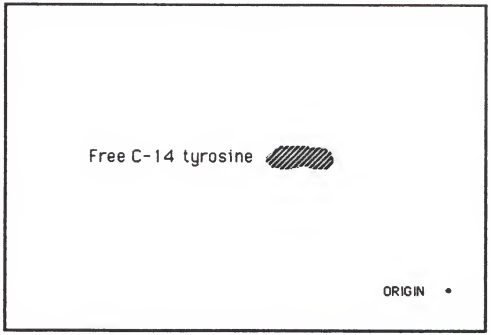

a.
Unknown C-14 tyrosine metabolite
II
Free C-14 tyrosine
I
TLC
ORIGIN
ELECTROPHORESIS
b.
Free C-14 tyrosine
I
TLC
ORIGIN
ELECTROPHORESIS

Figure 13. Analysis of C-14 tyrosine metabolites in the pharate adult whole body extracts of Periplaneta americana by 2-dimensional mapping. (a.) unhydrolyzed. (b.) hydrolyzed. = radiolabeled, and = radiolabeled < 200 counts/min.

a.


V
Unknown C-14 tyrosine
metabolite
II
VI
I
III
Free C-14 tyrosine
IV
ORIGIN
TLC
ELECTROPHORESIS

b.


Free C-14 tyrosine
ORIGIN
TLC
ELECTROPHORESIS

Hemolymph and whole body samples of P. americana pharate adults, injected with S-35 displayed only one major radiolabeled area (metabolite II) on the x-ray film (Fig. 14a, 15a). The labeled area was similar in mobility and solubility to Unk. IV, the tyrosine C-14 metabolite (metabolite II). The hemolymph sample (Fig. 14a) displayed three additional small and faint radioactive areas (metabolites III, IV, and V). Whole body sample exhibited six faint small areas (Fig. 15a metabolites III, IV, V, VI, VII, and VIII). Upon hydrolysis of both the hemolymph and whole body samples, there were no radioactive zones located on the x-ray film of either (Fig. 14b, 15b). These results indicate that there are at least four sulfate containing metabolites in hemolymph and at least seven found in the whole body. These sulfate conjugates may or may no be derived from tyrosine since no corresponding labeling was found in C-14 tyrosine experiments.

In unhydrolyzed extracts of adult P. americana (hemolymph and whole body) 1-12 hrs after ecdysis, several tyrosine C-14 metabolites were located on x-ray film (Fig. 16a, 17a). The radiolabeled area (metabolite I) corresponding to the tyrosine standard was the major area of radioactivity, all other areas were weak. Unk. IV was present (metabolite II) in both the hemolymph and whole body samples, but it was low in intensity like the other radiolabeled areas. There were four more areas of radiolabeled material in hemolymph and nine new areas in whole body extracts. Hydrolysis of both samples again yielded only one major

Figure 14. Analysis of S-35 metabolites in pharate adult hemolymph extracts of Periplaneta americana by 2-dimensional mapping. (a.) unhydrolyzed. (b.) hydrolyzed. ▨ = radiolabeled, ▦ = non-radiolabeled and ▩ = radiolabeled < 200 counts/min.

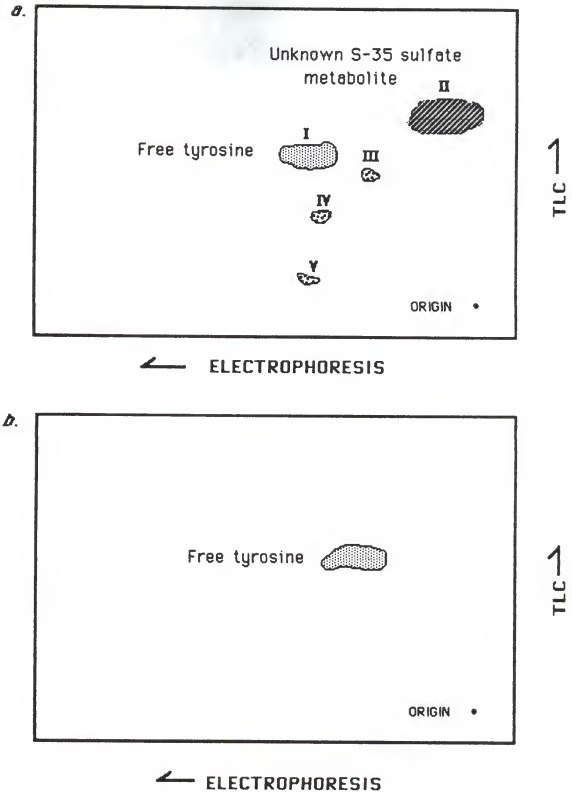

a.
Unknown S-35 sulfate metabolite
II
Free tyrosine
I
III
IV
V
ORIGIN
TLC
ELECTROPHORESIS
b.
Free tyrosine
ORIGIN
TLC
ELECTROPHORESIS

Figure 15. Analysis of S-35 metabolites in pharate adult whole body extracts of Periplaneta americana by 2-dimensional mapping. (a.) unhydrolyzed. (b.) hydrolyzed. = radiolabeled, = non-radiolabeled and = radiolabled < 200 counts/min.

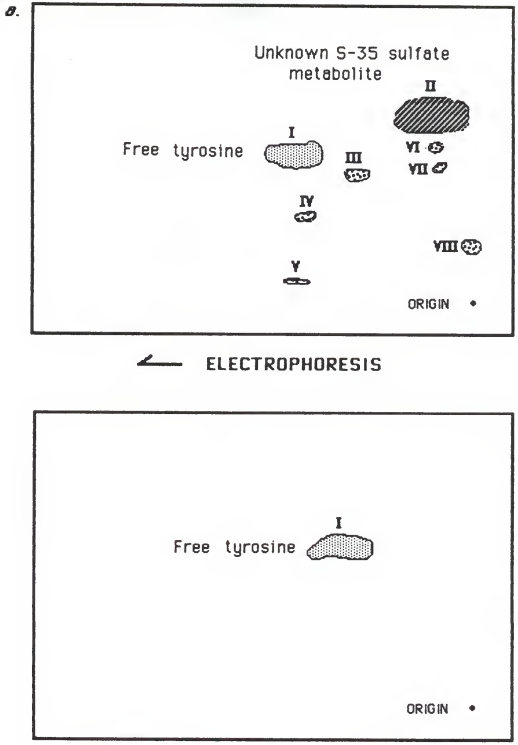
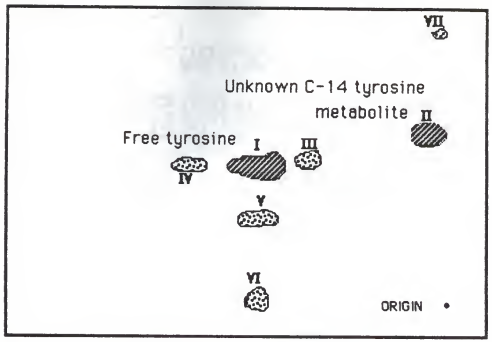

B.
Unknown S-35 sulfate metabolite
II
I
Free tyrosine
III
VI
VII
IV
VIII
V
ORIGIN
TLC
ELECTROPHORESIS
I
Free tyrosine
ORIGIN
TLC
ELECTROPHORESIS

Figure 16. Analysis of C-14 tyrosine metabolites in adult hemolymph extracts of Periplaneta americana by 2-dimensional mapping. (a.) unhydrolyzed. (b.) hydrolyzed. = radiolabeled and = radiolabeled < 200 counts/min.

a.


VII
Unknown C-14 tyrosine
metabolite
II
Free tyrosine
I
III
IV
V
VI
ORIGIN
TLC
ELECTROPHORESIS

b.

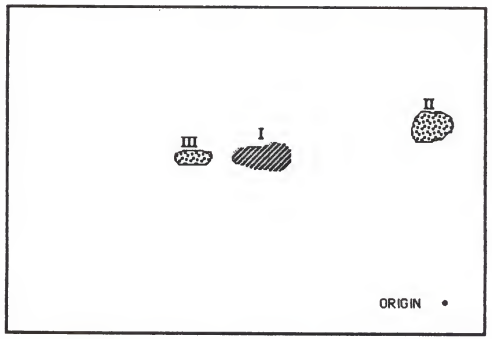
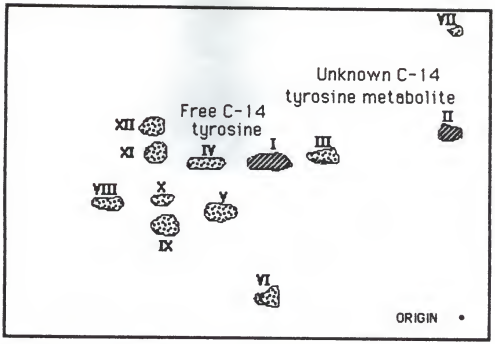
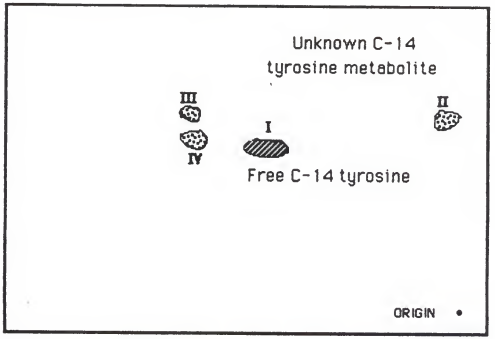

II
III
I
ORIGIN
TLC
ELECTROPHORESIS

Figure 17. Analysis of C-14 tyrosine metabolites in adult whole body extracts of Periplaneta americana by 2-dimensional mapping. (a.) unhydrolyzed. (b.) hydrolyzed. ▨ = radiolabeled and ▩ = radiolabeded < 200 counts/min.

a.


VII
Unknown C-14
tyrosine metabolite
Free C-14
tyrosine
XII
II
XI
IV
I
III
VIII
X
V
IX
VI
ORIGIN
TLC
ELECTROPHORESIS

b.


Unknown C-14
tyrosine metabolite
III
II
I
IV
Free C-14 tyrosine
ORIGIN
TLC
ELECTROPHORESIS

radioactive area, free tyrosine. There were however several minor areas of radioactivity remaining after hydrolysis in both hemolymph (metabolites II, III) and whole body (metabolites II, III, IV) (Fig. 16b, 17b).

The S-35 sulfate labeled whole body and hemolymph samples (unhydrolyzed) had several areas of radioactivity. The hemolymph samples had four zones of radioactivity (metabolites II, III, IV, V), while the whole body samples had these four metabolites plus one other (Fig. 18a, 19a). The area which corresponded with that of Unk. IV was greatly reduced in intensity when compared to the same area in the pharate adult stage. Hydrolyzed samples of both the hemolymph and whole body yielded no areas of radioactivity after fingerprint analysis (Fig. 18b, 19b). Hemolymph had at least four sulfate containing metabolites and whole body had five.

Identification of radiolabeled metabolites

Based on the information obtained from the TLC plates our initial hypothesis was that Unk. IV was tyrosine-O-sulfate or some tyrosine conjugate. Analysis by LCEC of the compounds contained in the radioactive zones showed that after hydrolysis dopamine was the only detectable compound. Samples from hemolymph of both C-14 tyrosine and S-35 sulfate injected cockroaches, removed from the area of unknown IV (metabolite II) and samples corresponding to the area of Unk. IV, taken from cockroaches not injected with radioactive materials (hemolymph),

Figure 18. Analysis of ^{35}S-35 metabolites in adult hemolymph extracts of Periplaneta americana by 2-dimensional mapping. (a.) unhydrolyzed. (b.) hydrolyzed. = radiolabeled, = non-radiolabeled and = radiolabeled < 200 counts/min.

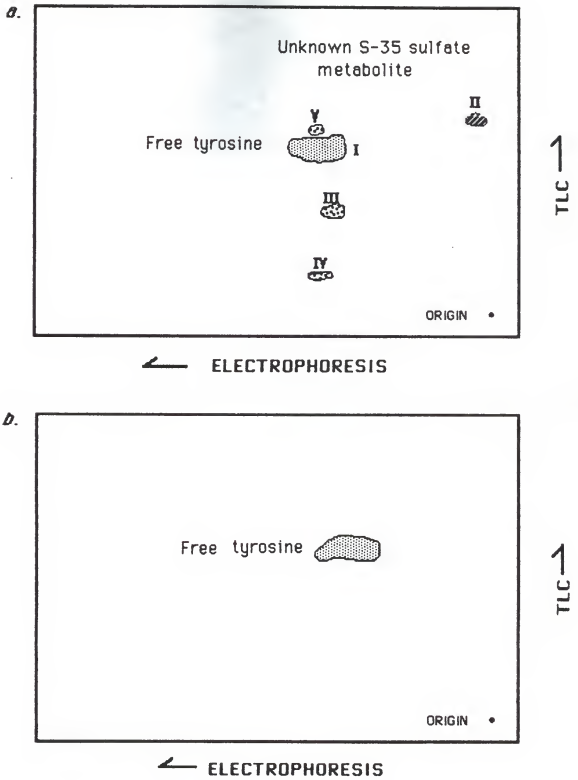
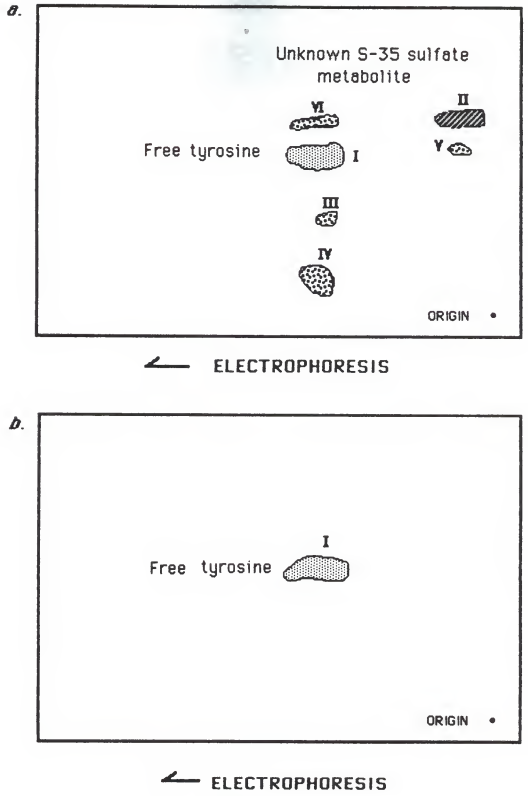

a.
Unknown S-35 sulfate metabolite
Free tyrosine
V
I
II
III
IV
ORIGIN
TLC
ELECTROPHORESIS
b.
Free tyrosine
ORIGIN
TLC
ELECTROPHORESIS

Figure 19. Analysis of S-35 metabolites in adult whole body extracts of Periplaneta americana by 2-dimensional mapping. (a.) unhydrolyzed. (b.) hydrolyzed. = radiolabeled, = non-radiolabeled and = radiolabeled < 200 counts/min.


a.
Unknown S-35 sulfate metabolite
VI
II
Free tyrosine
I
V
III
IV
ORIGIN
TLC
ELECTROPHORESIS
b.
I
Free tyrosine
ORIGIN
TLC
ELECTROPHORESIS

were hydrolyzed as before and were found to release dopamine as their major peak during LCEC analysis. This demonstrates that Unk. IV is probably dopamine-O-sulfate. Tentative evidence from LCEC analysis of both the 3-O and 4-O standards and actual hemolymph indicate that both dopamine 3-O-sulfate and dopamine 4-O-sulfate are present in the roach in an approximate 5-1 ratio (See Appendix B). Tyrosine sulfate and dopa-O-sulfate are potential metabolites in the hemolymph of P. americana. Unk. VI and VII in figure 15a could be indications of 35S labeled dopa-sulfate and tyrosine sulfate respectively. Unk. V could be dopa-sulfate in figure 19a. We have been unable to confirm either compound at the present time.

IV. DISCUSSION

Tyrosine storage

Tyrosine is the major precursor of catecholamines and their corresponding quinones that are utilized in the sclerotization, melanization and stabilization of insect cuticle. Tyrosine can be gained from the diet or by the hydroxylation of phenylalanine (reviewed by Brunet, 1980). There are several possible storage forms of tyrosine in insects. Tyrosine and its precursor phenylalanine are stored as peptides in certain species of Diptera (Bodnyark, 1978). Tyrosine is also stored in vacuoles that occur in the fat body of larval Calpodes ethlius and Manduca sexta. The form of tyrosine in these vacuoles is uncertain, but it may occur as free tyrosine crystals (McDermid and Locke, 1983). Tyrosine-O-phosphate is the storage molecule in Drosophila melanogaster (Mitchell and Lunan, 1964), while tyrosine glucoside (B-D-glucopyranosyl-O-L-tyrosine) is the storage molecule in Drosophila busckii (Chen et al., 1978), Manduca sexta and several other species of Lepidoptera, (Kramer et al., 1980; Ishizaki and Umebachi, 1980; Isobe et al., 1981; Lu et al., 1982), and Ceratitis capitata (Psarianos et al., 1985). The mechanism of tyrosine storage in cockroaches is unknown, but it may be stored in a tyrosine-rich protein in nymphal cockroaches. Arylphorins are a class of proteins that contain a relatively high content of tyro-

sine and phenylalanine (Telfer et al., 1983). Arylphorins have been identified in several orders, including Diptera (Munn et al., 1971), Lepidoptera (Tojo et al., 1980; Kramer et al., 1980), Hymenoptera (Ryan et al., 1984) and Dictyoptera (Duhamel and Kunkel, 1983). A larval-specific protein (LSP), high in tyrosine content, has been identified in B. orientalis (Kunkel, 1975) and was earlier shown to have cross-immunization reactions throughout the order (Kunkel and Lawler, 1974). LSP titers have been shown to increase before ecdysis of the last nymphal instar and it becomes the most abundant protein in the hemolymph of B. orientalis (Duhamel and Kunkel, 1978). This protein reaches peak concentrations 24-48 hours prior to nymphal-adult ecdysis (Kunkel, 1975; Duhamel and Kunkel, 1978). LSP titers decline during apolysis and then drop sharply at nymphal-adult ecdysis. By 24 hours after ecdysis, the concentration of LSP was barely detectable (Duhamel and Kunkel, 1978). Free tyrosine concentrations in the hemolymph of P. americana and L. maderae are very low prior to new cuticle formation, and follow a cyclical pattern in the hemolymph, fat body, and integument. Tyrosine sharply increases after apolysis, reaches a peak at nymphal-adult ecdysis, and then sharply declines following ecdysis (Wirtz and Hopkins, 1974; 1977a). Results also showed that phenylalanine and tyrosine do not accumulate as free amino acids in these cockroaches and that one or both of them may be stored in some type of storage molecule (Wirtz and Hopkins, 1977a).

Catecholamine storage

In this study a conjugate of tyrosine, such as a glucoside, has not been found in the either cockroach species. The possibility that tyrosine sulfate is a major soluble storage form of tyrosine was not supported by radiolabeling of metabolites with sulfur-35. Instead a catecholic conjugate, dopamine-O-sulfate was found to be sequestered during nymphal development. Dopamine-O-sulfate begins to accumulate in the hemolymph approximately 1-3 days after ecdysis of the last instar. This sulfate conjugate reaches peak titers near nymphal-adult apolysis, followed by a decline during the late pharate adult stage until ecdysis occurs. Dopamine-O-sulfate is also found in L. maderae. The arylphorin LSP in B. orientalis displays a similar pattern. Tyrosine that is incorporated in the arylphorin LSP protein may be the major source that is needed for the synthesis of catecholamines in P. americana, provided that tyrosine is released from this protein when needed for sclerotization. The catecholamine dopamine is stored as a sulfate conjugate in P. americana until it is required for sclerotization metabolism. Both the sulfate and arylphorin types of storage are apparently needed for sclerotization, melanization and stabilization of insect cuticle. The sequestration of dopamine-O-sulfate probably occurs so that it may be transported to the cuticle for tanning. It may be further metabolized in the hemolymph of the cockroach, at ecdysis, into NADA or NBAD and

then transported to the cuticle, but this is unlikely. Sulfation no doubt renders the ring less reactive toward oxidative enzymes. Sulfate conjugation is, therefore, a mechanism for both protection and transport of dopamine to the cuticle. Little, if any of the sulfate moiety was found to be transported into the cuticle of tanning P. americana (Bodnyark and Brunet, 1974). The sequestration of tyrosine in arylphorin LSP and subsequent release at ecdysis appears to enable P. americana and L. maderae to store large amounts of tyrosine that are needed for mobilization during catecholamine synthesis. Tyrosine hydroxylase and DOPA decarboxylase activity reach peak levels at 6 hours post-ecdysis, followed by a decline to pre-ecdysial levels, probably for metabolizing the tyrosine liberated from the LSP or some other type of tyrosine storage molecule.

Although both tyrosine hydroxylase activity and dopa decarboxylase activity are low in last instar P. americana before ecdysis (Hopkins and Wirtz, 1976; Wirtz and Hopkins, 1977b; Hopkins, 1982;), enough enzymes activity may be present during this period to hydroxylate tyrosine to DOPA and decarboxylate DOPA to dopamine. Dopamine would then accumulate as the protected sulfate conjugate. It is unknown at present whether DOPA is sulfated or not. It may be one of the minor sulfate metabolites observed on the TLC plates. Dopamine-O-sulfate is probably not hydrolyzed to any great extent until after ecdysis.

The decrease of the dopamine-O-sulfate concentrations during late pharate adult stage may be due to two possible reasons. One explanation is that the increase in hemolymph volume that occurs prior to ecdysis (Mills and Whitehead, 1970) might dilute the concentration of dopamine-O-sulfate. The levels of tyrosine hydroxylase, DOPA decarboxylase and dopamine sulfatase may not be high enough during this period of time to maintain a high concentration of dopamine-O-sulfate during the increase in hemolymph volume. The second explanation is that the dopamine-O-sulfate maybe transported from the hemolymph to some other tissue, possibly the fat body or epidermis.

After ecdysis, the concentration of dopamine increases due to increased enzyme activity. The LSP titers in B. orientalis start to decline at a similar time to that of the dopamine-O-sulfate in P. americana, but this decline is not due to the increase in hemolymph volume (Duhamel and Kunkel, 1978). LSP titers in the hemolymph rapidly decline immediately after ecdysis, probably due to the action of proteolytic enzymes or transportation from the hemolymph to other tissues.

Role of dopamine-O-sulfate

In P. americana, the majority of the dopamine-O-sulfate in the hemolymph may be transported to the epidermis and/or cuticle where it is hydrolyzed, and dopamine subsequently acylated to yield NADA and NBAD. As stated earlier, the sulfate moiety does not accompany dopamine into

the cuticle. The decrease of dopamine in the hemolymph, with no corresponding increase of the other catecholamines in the hemolymph such as NADA or NBAD, supports this hypothesis. Free dopamine is found in the cuticle of P. americana several hours after ecdysis, indicating that dopamine is being transported into the cuticle. Metabolism to NADA and NBAD could, however, be occurring in the hemolymph, but to a minor extent relative to that in cuticle. The rate of transport of these catecholamines, synthesized in the hemolymph, to the cuticle would have to occur so rapidly that there would be no build-up of these catecholamines in the hemolymph.

Catecholamines in cuticle and their role in sclerotization

NADA and NBAD are the major substrates (after metabolism from dopamine) involved in the sclerotization of new cuticle. In the past, NADA has been considered the principal catecholamine metabolite in the sclerotization of insect cuticle (reviewed by Brunet, 1980), while NBAD was more recently identified as a major metabolite in the sclerotization of pupal cuticle in Manduca sexta (Hopkins et al., 1982; 1984). Cuticle hardness and pigmentation probably depend on a complex pattern of catecholamine synthesis and utilization (Hopkins et al., 1982; 1984; Kramer et al., 1984). NADA has been associated with colorless hard cuticle, NBAD with brown hard cuticle and dopamine has been associated with the production of melanin (Hopkins et al., 1982; 1984; Kramer et al., 1984).

Since P. americana cuticle appears to be a hard brown cuticle, NBAD would be expected to be the major catecholamine in its cuticle. While NBAD is not the major catecholamine in the hemolymph of P. americana, it is a major catecholamine in its cuticle. NADA may also be considered a major catecholamine in the cuticle. NADA can be metabolized to NANE and NBAD to NBANE through B-hydroxylation in the cuticle. NBANE is the most abundant catecholamine in the cuticle of P. americana. This evidence tends to support the theory that specific catecholamines and their concentrations are related to different types of sclerotized cuticle (Hopkins et al., 1984). The increase in the B-alanine containing catecholamines confirms the increase of B-alanine in P. americana that was observed in hydrolyzed cuticle (Kennaugh, 1958; Hackman and Goldberg, 1971). B-Alanine was not present in newly ecdysed cuticle, but was found to increase after ecdysis, reaching a plateau several hours after ecdysis.

Accumulation of free catecholamines in the cuticle

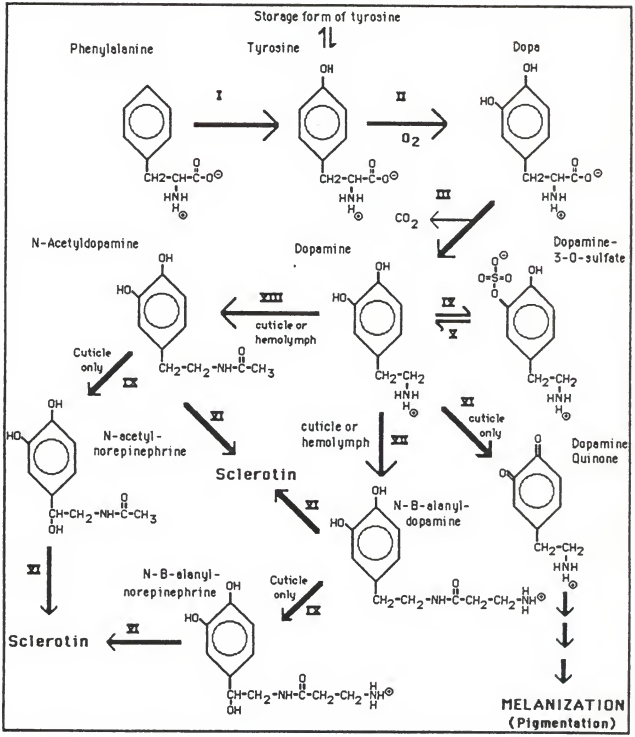
The accumulation of large amounts of these catecholamines in insect cuticle suggests a possible storage system (Hopkins et. al., 1984). The catechols could then be available for wound healing or additional sclerotization. It has been shown that M. sexta continues to darken for several days after ecdysis (Hopkins et al., 1984). In Schistocera gregaria, sclerotization has been demonstrated to continue for several

weeks after adult ecdysis (Andersen and Barrett, 1971; Andersen, 1974). A final possibility is that these catecholamines help stabilize cuticle by dehydration, in which water molecules are replaced by diphenols (Vincent and Hillerton, 1979; Hillerton and Vincent, 1979). This would account for the accumulation of catecholamines in the cuticle of P. americana.

Proposed catecholamine synthesis pathway in the cockroaches

The proposed pathway for the synthesis of catecholamines for utilization in cuticular stabilization and pigmentation in P. americana and other cockroaches is shown in Fig. 20. In newly ecdysed and mature adults of P. americana, tyrosine was found to be the major metabolite of phenylalanine via hydroxylation (Murdock et al., 1970a). Tyrosine could be sequestered in a arylphorin LSP storage molecule until nymphal-adult ecdysis occurs (Kunkel and Lawler, 1974; Kunkel, 1975; Duhamel and Kunkel, 1978; 1983). Free tyrosine that is not assimilated into the LSP during the last instar is hydroxylated to DOPA by tyrosine hydroxylase or tyrosinase (Hopkins, 1982). DOPA decarboxylase then converts dopa to dopamine (Hopkins and Wirtz, 1976; Wirtz and Hopkins, 1977b), which is then sulfated by a sulfotransferase and stored until ecdysis. It is unknown at this time whether minor pathway occurs where DOPA is sulfated and also stored until ecdysis. At ecdysis tyrosine could be released from a storage molecule (LSP) via proteolytic enzymes and subsequently

Figure 20. Proposed pathway for catecholamine biosynthesis in Periplaneta americana. I = phenylalanine hydroxylase, II = tyrosinase or tyrosine hydroxylase, III = dopa decarboxylase, IV = sulfotransferase, V = sulfate hydrolase, VI = tyrosinase, VII = N-B-alanyldopamine synthetase, VIII = N-acetyldopamine synthetase, IX = B-hydroxylase.


Storage form of tyrosine
Phenylalanine
Tyrosine
Dopa
I
II
O_2
III
CO_2
N-Acetyldopamine
Dopamine
Dopamine-3-O-sulfate
XIII
cuticle or hemolymph
IX
X
Cuticle only
XII
N-acetyl-norepinephrine
XI
cuticle or hemolymph
VII
cuticle only
Dopamine Quinone
Sclerotin
VI
N-β-alanyl-dopamine
N-β-alanyl-norepinephrine
Cuticle only
IX
Sclerotin
VI
MELANIZATION
(Pigmentation)

hydroxylated and decarboxylated to yield dopamine. Dopamine is then conjugated with sulfate to form dopamine-O-sulfate, which maybe transported into the epidermis or other tissue for cleavage of the sulfate. In the epidermis or cuticle, dopamine may be acylated with acetate or B-alanine to form NADA or NBAD respectively. Dopamine may also be oxidized to form dopamine quinone as a precursor in the production of melanin (Kramer et al., 1983; Aso et al., 1984). The melanin pathway appears to be more prevalent in L. maderae cuticle than in P. americana cuticle. Newly ecdysed L. maderae darken the cuticle with melanistic patterns before sclerotization while no observable melanin is deposited in P. americana cuticle (Hopkins, unpublished observations). the latter.

NADA and NBAD may be converted to NANE and NBANE, respectively, by hydroxylation of the B-carbon in the cuticle. These four catecholamine metabolites may be further oxidized to quinones for crosslinking the chitin-protein matrix of the cuticle either through quinone tanning or B-sclerotization. In P. americana, the metabolism of dopamine to NBAD and NBANE is thought to be the major synthetic pathway for tanning agents in newly ecdysed cockroaches. After approximately 24 hours the synthesis of NADA also becomes significant in P. americana, but the majority of synthesis is towards NBAD and NBANE. The synthesis of melanin in the American cockroach has not been shown to occur. In L. maderae, both the synthesis of NADA in the hemolymph and the production of melanin appear to be major pathways. This interpretation is based on

preliminary hemolymph and cuticle catecholamine data and observations on the pigmentation that occurs very rapidly in L. maderae cuticle after ecdysis.

Hormones in Periplaneta americana and their proposed roles

The regulation of molting, metamorphosis and sclerotization are mediated by insect hormones. Ecdysone and juvenile hormones (JH) are associated with regulating molting and metamorphosis, but the regulatory mechanism is more complex than a two-hormone model (reviewed by Jungreis, 1979). The release of the hormone bursicon is needed for the initiation of cuticle sclerotization (Fraenkel and Hsiao, 1965).

Titers of ecdysone and JH have been studied during the last nymphal instar of P. americana (Shaaya, 1978). JH titers are high enough only during the second quarter (approx. age 10 days) of the last instar to stimulate the production of glucosides in decapitated females and are insufficient in the other quarters to initiate this metabolism. Ecdysone was not detected during the first 20 days of the last instar. Ecdysone titers began to rise after 20 days reaching a plateau at approximately 30 days after ecdysis. Ecdysone titers then began to rise at about day 35, reaching a peak shortly before ecdysis and declining rapidly at ecdysis. JH titers are at their lowest when ecdysone was released (Shaaya, 1978). Ecdysteroids in adult P. americana show a

continued decline in titer after ecdysis (Weaver et al., 1984). The major ecdysteroid in P. americana was 20-hydroxyecdysone.

The way in which bursicon stimulates the sclerotization of insect cuticle is not precisely known, but one mechanism probably involves the transport of tyrosine into hemocytes by altering the permeability of the hemocyte membranes (Mills and Whitehead, 1970). Bursicon has been shown to induce adenyl cyclase activity to form cyclic adenosine 5'-monophosphate (cAMP) in P. americana (Compton and Mills, 1982), while bursicon-induced tanning has been shown to be mimicked by cAMP in P. americana (Vandenberg and Mills, 1974).

Bursicon is released from neurohaemal organs located posteriorly to the terminal abdominal ganglion (last three ganglion) in P. americana (Mills et al., 1965; Mills, from Bell and Adiyodi, 1982). Bursicon is released into the hemolymph shortly after ecdysis and reaches maximum titers 90 minutes later and then slowly declines until disappearance 8 hours after ecdysis (Mills, 1966). Bursicon is released at ecdysis in L. maderae. It reaches peak titers at 1.5 hours after ecdysis and disappears by 3 hours after ecdysis. The site of release of bursicon in L. maderae is thought to be the thoracic ganglion (Srivastava and Hopkins, 1975).

The interactions of amino acids, catecholamines and their conjugates, proteins, enzymes and hormones form a complex system in regulat-

ing the development and metamorphosis of P. americana. LSP data is for B. orientalis.

The high levels of JH during the middle of the instar (Shaaya, 1978) may stimulate the sequestration of dopamine-O-sulfate as there is a sharp increase in conjugate at this time. The increase in LSP titers at the same time (Duhamel and Kunkel, 1983) may also be controlled by JH. As JH titers decrease in the later part of the stadium, ecdysone titers (Shaaya, 1978) increase to a plateau as does the LSP titers. A second pulse of ecdysone near the end of the stadium correlates with a rapid increase of LSP titers during the 4th quarter. Dopamine-O-sulfate also shows a marked increase late in the last stadium.

Dopamine-O-sulfate, ecdysone, LSP all reach peak titers at apolysis, while JH titers are at their lowest. At apolysis, antidiuretic activity increases causing an increase in hemolymph volume (Mills and Whitehead, 1970). After reaching peak titers at the onset of apolysis, ecdysone declines rapidly through ecdysis. Only female P. americana exhibit a small increase in ecdysone after it reaches its lowest titers at 5 days after ecdysis. This peak occurs 8 days after ecdysis (Weaver et. al, 1984). Dopamine-O-sulfate and LSP also show declines during this time. Ecdysone and LSP decline can not be explained by the rising hemolymph volume in the cockroach (Shaaya, 1978; Duhamel and Kunkel, 1978). The decline in dopamine-O-sulfate may be explained by the rising hemolymph volumes.

Free tyrosine increases rapidly during apolysis (Wirtz and Hopkins, 1974; 1977a). This may be due to tyrosine release from the LSP or some other tyrosine storage form via proteolytic enzymes. Ecdysone may be responsible in activating the proteolytic enzymes that release tyrosine from the LSP or other storage molecules. Dopa decarboxylase activity increases after apolysis and reaches peak activity 6 hours after ecdysis (Wirtz and Hopkins, 1978). Free tyrosine titers reach their maximum concentrations at ecdysis, corresponding to the lower levels of the LSP. Ecdysone and JH are at their lowest levels at ecdysis. At ecdysis, tyrosine hydroxylase activity increases dramatically, causing a rapid drop in free tyrosine titers. LSP experiences a rapid decline at ecdysis as well. Dopamine and dopamine-O-sulfate titers peak at 1 hour after ecdysis and then decline rapidly.

Bursicon is released at ecdysis and titers are highest 90 minutes later followed by a gradual decline to disappearance 8 hours after ecdysis (Mills, 1966). Bursicon may activate several processes, such as, the transport of tyrosine into the hemocytes, synthesis or activation of the enzyme tyrosine hydroxylase or tyrosinase, and the transport of dopamine-O-sulfate into the epidermis or other tissue. Dopamine and dopamine-O-sulfate titers drop dramatically after the bursicon peak.

Species comparison of catecholamines in cockroaches

The type and amounts of catecholamines differ among cockroach species. This difference may be due to the evolution of protective coloration and for the physical properties of the exoskeleton peculiar to each species. In L. maderae, the release of a repugnant odor when disturbed and camouflage are the chief means of protection. L. maderae is a sub-tropical cockroach and lives in a humid environment in forest detritus. The production of melanin causes the dark coloration of the cuticle and thus allowing the cockroach to blend in with surrounding environment. P. americana is a world-wide species that is able to live in climates of lower humidity than that L. maderae. The American cockroach relies on rapid escape behavior for protection when threatened. Cuticular stabilization probably occurs more rapidly in P. americana to minimize water loss and allow the return to rapid locomotion through hardening of the exoskeleton.

In comparing other species of cockroaches to P. americana and L. maderae (Table 3), one can see some surprising differences in the types and quantities of catecholamines in the hemolymph. A major difference occurs in the amounts of catechols in the species surveyed. L. maderae has a very low level of dopamine at ecdysis (0.015 mM), but it exhibits a relatively high NADA concentration compared to other cockroaches (0.15 mM). The oriental cockroach, B. orientalis, a member of the same subfamily as P. americana, has a black cuticle even darker than L. maderae.

One might expect its catecholamine profile to be similar to L. maderae due to the coloration of the cuticle. This is not the case as B. orientalis has a profile similar to P. americana with dopamine being the major catechol (0.25 mM) (Table 5). As cuticle tanning progresses in B. orientalis, NBAD becomes a major catecholamine in the hemolymph, unlike any other cockroach species surveyed.

In comparison of the normal B. germanica and its pale mutant there is over a 10-fold difference in the amount of dopamine present in the hemolymph at ecdysis with the pale mutant having the least. B. germanica normally has several black markings on its cuticle, especially the pronotum. These markings are not seen in the pale mutant. Since dopamine is a precursor for the production of melanin, the lack of this catechol would prevent melanin formation. The pale mutant has low levels of dopamine in its hemolymph at ecdysis compared to the normal cockroach. This lack of dopamine could be responsible for the missing black markings on the pronotum of the pale mutant B. germanica.

Comparison of catecholamines with other species

When comparing the catechol content of cuticle from P. americana and L. maderae to that of cuticle from other insect species, we can see differences in the catechols and their titers that may determine, in part, the type of cuticle formed. M. sexta pupal cuticle and P. ameri-

cana adult cuticle have high concentrations of NBAD and its metabolite NBANE (Hopkins et al., 1984). NBANE and NBAD are thought to be the major catecholamines in cuticles, that are predominantly dark brown. It is not known what proportion of these catecholamines are used to sclerotize or pigment cuticle of either species. In the larvae and adult of M. sexta NADA is the major catecholamine in clear or light colored cuticle. In the red flour beetle, T. castaneum, both the wild type and black mutant have high levels of NADA (Kramer et al., 1984). During eclosion, elytral cuticle accumulates much higher amounts of NBAD in the wild type than in the black mutant. The black mutant has very low levels of NBAD and high levels of dopamine compared to the wild type. The excess free dopamine is oxidized to form melanin in the black mutant while typical brown cuticle is formed in the wild type elytra. The black mutants can be rescued with an injection of NBAD, that enables them to produce the normal rust-reddish color cuticle. The wild type has rust reddish cuticle that, in color, is similar to P. americana.

In Diptera, M. autumnalis uses minerals, such as calcium and magnesium, rather than catechols to stabilize its puparium and thus has low levels of catechols. Dopamine is the primary catechol in the face fly puparial cuticle which is gray white in appearance. The puparium of M. domestica and S. calcitrans is similar in color to P. americana, but NBAD is the predominant catechol in these species of flies (Roseland et al., 1985).

Concluding remarks

The interplay of catecholamines in the sclerotization of cuticle has become a much more complex phenomenon then originally proposed. This research has added further evidence to support the hypothesis that the type and amount of catecholamines determine the type of cuticle formed. Further research is now needed to answer several questions concerning the sclerotization of insect cuticle. These are: 1. What occurs when catecholamines are incorporated (metabolized) into the chitin-protein matrix? 2. What enzymes and hormones are involved in this supermolecular assembly and how are they regulated? 3. Can the enzymes and hormones be influenced by insect growth regulators? 4. Is the tyrosine stored in an arylphorin LSP utilized for the stabilization and melanization in cockroach cuticle? 5. Why does B. orientalis have NBAD associated with black cuticle whereas dopamine is associated with the black mutant of T. castaneum? 6. What are the physical property differences among types of cuticles formed? This is an incomplete list of the unanswered questions concerning the complex role of catecholamines in the stabilization and pigmentation of cuticle. More research is needed to determine if a method of insect control can be established by using the knowledge of how catecholamines and their metabolites work in the stabilization and melanization of insect cuticle.

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VI. APPENDIX

A.

This experiment was conducted to determine if the freezing of the cockroaches had any effect on the amount of catecholamines recovered. Since dopamine is the most abundant catecholamine in the hemolymph of Periplaneta americana, it was chosen as the catechol to be observed. Three cockroaches were taken at three different developmental times. They were anesthetized with CO_2 and bled by cutting off the left front leg. The hemolymph (10 ul) was immediately transferred into a vial containing the internal standard. The cockroach was then quick-frozen in dry ice, held 24 hours at -20^{o} C, thawed in a desicator and hemolymph (10 ul) collected and transferred into a vial containing the internal standard. Hydrolysis of all samples and recovery of the catechols from alumina extraction were conducted. Samples were then analyzed by LCEC as previously discussed in Methods and Materials. Statistical analysis was done by a random block design blocking on time. The results and conclusions are stated below.

	TIME FRAME				
	BEFORE ECDY.	1 HOUR AFTER ECDY.	6 HOURS AFTER ECDY.	TOTAL	MEAN
DATA uM/l					
FREEZING	60.71	57.69	1.42	119.82	39.94
NO FREEZE	80.57	58.44	1.62	140.33	46.78
TOTAL	141.28	115.83	3.04	260.15	

C= 11279.67

SOURCE	D.F	SS	MS	F-TEST
TIME	2	5413.27	2706.63	
TREATMENT	1	70.11	70.11	1.102
RESIDUAL	2	127.23	63.61	
TOTAL	5	5610.60		

Based on the F-Test value of 1.102, there is not enough information to state that there is a significant difference in the two treatments, freezing and non-freezing. Therefore the primary method of collecting non-clotting hemolymph by rapid freezing of cockroaches in dry ice did not result in dopamine levels significantly different from the hemolymph collected from live insects.

B.

This experiment was conducted to determine whether the dopamine-O-sulfate was dopamine-3-O-sulfate or dopamine-4-O-sulfate. Standards of both dopamine-O-sulfates were obtained from Dr. J. Stephen Kennedy, National Institute of Mental Health, Rockville, Maryland and were analyzed on a LCEC system using a 100% methanol mobile phase and the electrochemical detector and column as previously described. Dopamine-3-O-sulfate had a retention time of 4.8 minutes and dopamine-4-O-sulfate 4.4 minutes which provided adequate separation. Hemolymph was collected from P. americana cockroaches at early apolysis as described previously with the exception that 1 M acetic acid was used instead of 1.2 M HCl. The sample was then centrifuged as before and then directly injected into the LCEC. Approximately 9 major and 3 minor peaks were detected with two having similar retention times to both dopamine sulfates. Co-injection of both sample and standards provided evidence that both dopamine-3-O-sulfate and dopamine-4-O-sulfate are present in the hemolymph of P. americana. Dopamine-3-O-sulfate is approximately 5 times greater than dopamine-4-O-sulfate based upon these results.

CATECHOLAMINES IN THE HEMOLYMPH AND CUTICLE OF THE AMERICAN COCKROACH, *PERIPLANETA AMERICANA* (L.) AND THE MADEIRA COCKROACH, *LEUCOPHAEA MADERAE* (F.)

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Abstract

Catecholamines were analyzed by liquid chromatography with electrochemical detection the hemolymph and cuticle of Periplaneta americana and Leucophaea maderae during the last nymphal and adult stages. Dopamine sulfate was found to be the major catecholamine in the hemolymph of P. americana during nymphal development and adult apolysis and ecdysis. Approximately 80% of the dopamine in the hemolymph of the American cockroach is sequestered as the sulfate ester. N-Acetyldopamine (NADA) was the major catecholamine in L. maderae at nymphal-adult ecdysis, although substantial amounts of dopamine sulfate was also present. N-B-Alanyldopamine (NBAD) was less abundant than either dopamine or NADA in hemolymph, but was a major catechol in the cuticle of both species. An unknown electroactive metabolite of tyrosine was present in the hemolymph of P. americana throughout development but showed little correlation with cuticle tanning. Dopamine sulfate concentrations in P. americana were low in the early last instar but increased to peak titers just before larval-adult apolysis (0.95 mM). Dopamine sulfate declined during apolysis, increased again at ecdysis, Dopamine rapidly declined during cuticle tanning. NADA concentrations reached peak titers 6 hours after ecdysis (0.25 mM) in L. maderae.

N-B-Alanyldopamine (NBAD) and N-B-alanylnorepinephrine (NBANE) were the major catecholamines found in the cuticle of P. americana (2.1 uM/g and 2.5 uM/g respectively). Both were observed to increase as the cuticle tanned. NBANE (2.0 uM/g) was the most abundant catechol in the cuticle of L. maderae with NADA (0.26 uM/g) and N-acetylnorepinephrine (NANE) (0.26 uM/g) being the next most abundant.

Dopamine was the major catecholamine in the hemolymph of several cockroach species at larval-adult ecdysis: Blatta orientalis, Blattella germanica, Gromphadorhina portentosa, and Blaberus craniifer. Cockroaches, as a group, appear to store large amounts of dopamine, probably as sulfate esters, during development for tanning of cuticle. Dopamine-3-O-sulfate is predominant but the 4-O-sulfate is also present in P. americana. The dopamine is then acylated with acetate or B-alanine to form NADA and NBAD respectively which increase in cuticle during tanning and probably reacts with the chitin-protein matrix for stabilization. The acylated norepinephrine derivatives (NBANE and NANE) also accumulate in tanning cuticle and they likely have an important role in the sclerotization process.